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Carbonyl stress and a combination of astaxanthin/vitamin C induce biochemical changes in human neutrophils

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ABSTRACT

The purpose of the present study was to find out whether co-treatment of human neutrophils with high glucose and methylglyoxal (MGO) can alter the biochemical parameters of human neutrophils. We also examined if astaxanthin associated with vitamin C can improve those biochemical parameters. Neutrophils from healthy subjects were treated with 20 mM of glucose and 30 μM MGO followed or not by the addition of the antioxidants astaxanthin (2 μM) and vitamin C (100 μM). MGO/high glucose treatment reduced the phagocytic capacity and the G6PDH, total/SOD and GR activities. Additionally, there was an increase in the activity of myeloperoxidase (MPO) with consequent increase in the hypochlorous acid production, CAT activity and in the release of IL-6 cytokine without changes in intracellular calcium mobilization. Our study also shows that the association of astaxanthin with vitamin C greatly improved neutrophil phagocytic capacity, decreasing all reactive oxygen species measured, pro-inflammatory IL-1β and TNF-α release, MPO activity and HClO production. The combination of astaxanthin with vitamin C alone has more antioxidant and anti-inflammatory effects than when they were in the presence of MGO/high glucose. Injury to the function of neutrophils due to high glucose and methylglyoxal appears not to involve oxidative stress or calcium release. The association of antioxidants astaxanthin and vitamin C promoted a significant improvement in the function of neutrophils and in the redox status.

1. Introduction

Methylglyoxal (MGO), a highly reactive dicarbonyl metabolite produced during glucose metabolism, is a major precursor of the advanced glycation end products (AGEs). AGEs are the result of the non-enzymatic glycation of proteins/lipids which accumulate during natural aging. In general, they are also greatly augmented in disorders such as diabetes, renal failure and Alzheimer's disease (Brownlee, 1995; Schmidt et al., 1994; Takedo et al., 1996). MGO clinical significance is based on the fact that there is a strong association between the pathophysiology of type 2 diabetes along with associated vascular and neuronal complications, and increased plasma MGO and AGEs concentrations (Turk, 2010). Dhar et al. (2008) showed that vascular smooth muscle cells treated with high glucose (25 mM) increased intracellular MGO concentration accompanied by increased oxidative stress. Both MGO and high glucose may activate different pathways, increasing reactive species of oxygen and nitrogen production (ROS/RNS) which in turn, leads to oxidative stress (Wang et al., 2009). AGEs formed from high glucose and/or MGO can also link to specific AGE-receptor (RAGE) present in the plasma membrane of different cell types, including immune cells, and trigger inflammatory response by increasing activation of NFκB signaling pathway (Kalapos, 1999).

Immune cell dysfunction is a common feature involved in the pathogenesis and/or late complications of several chronic diseases. Phagocytosis and killing of the pathogens are the primary functions of neutrophils in the innate immune response in order to contain and kill invading microbial pathogens. This process is achieved through a series of rapid and coordinated responses (Fialkow et al., 2007). Neutrophils exhibit a potent antimicrobial arsenal that includes oxidants, proteinases, and antimicrobial peptides. Neutrophils also produce prodigious quantities of ROS and RNS such as superoxide and nitric oxide through the activity of oxidant-generating systems such as the phagocyte NADPH oxidase (Sheppard et al., 2005) and nitric oxide synthase (NOS), respectively (Fialkow et al., 2007; Gebska et al., 2005; Kleinert et al., 2004).

Astaxanthin (ASTA) is an orange-reddish carotenoid pigment found in living organisms particularly in the marine environment where it is present in microalgae, plankton, krill and seafood. It gives salmon, trout, and crustaceans such as shrimp and lobster their distinctive pinkish coloration (Fassett and Coombes, 2011). ASTA belongs to the xanthophyll class of carotenoids and is closely related to b-carotene, lutein, lycopene, and zeaxanthin, sharing

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with them many of health-promoting effects attributed to carotenoids (Yuan et al., 2011). Several studies have demonstrated that astaxanthin exhibits a wide variety of biological activities, including the prevention and treatment of various diseases, such as cancers, chronic inflammatory diseases, metabolic syndrome, diabetes, diabetic nephropathy, cardiovascular diseases, gastrointestinal diseases, liver diseases, and neurodegenerative diseases (Chew et al., 1999; Jyonouchi et al., 2000; Kishimoto et al., 2010; Marin et al., 2011; Naguib, 2000; Otton et al., 2011).

The presence of the hydroxyl and keto moieties on each ionone ring (Fig. 1) explains some of its unique features such as the ability to be esterified, a higher antioxidant activity, and a more polar nature than other carotenoids (Hussein et al., 2006). Astaxanthin may act as a strong antioxidant by donating the electrons and reacting with free radicals to convert them into more stable products and terminate free radical chain reaction in a wide variety of living organisms. The nonpolar middle segment of the astaxanthin molecule is a series of carbon-carbon double bonds, which alternate with carbon-carbon single bonds, termed "conjugated". This polar-nonpolar-polar layout also allows the astaxanthin molecule to take a transmembrane orientation, making a precise fit into the polar-nonpolar-polar span of the cell membrane (Kidd, 2011). As mentioned by many authors, the antioxidant activity of astaxanthin appears to be greater than that of beta-carotene and alpha-tocopherol (Fukuzawa et al., 1998; Naguib, 2000). However, studies from our group which evaluated the antioxidant effect of astaxanthin on leukocytes in human and animal models, showed a modest antioxidant action (Bolin et al., 2010; Guerra and Otton, 2011; Macedo et al., 2010; Mattei et al., 2011; Otton et al., 2010, 2011), mainly observed in the reduction of superoxide and hydrogen peroxide production.

Vitamin C is an essential micronutrient, which has been implicated in a variety of biological processes, including immune response (Maeng et al., 2009). Vitamin C or L-ascorbic acid is the body's most important intracellular and extracellular aqueous-phase antioxidant. This antioxidant easily scavengers peroxyl radicals, superoxide anion, singlet oxygen and hypochlorite (Sies and Stahl, 1995). The oxidation of vitamin C by reacting with ROS generates the ascorbyl radical that has little reactivity, crucial to the antioxidant effect of vitamin C. Ascorbic acid is considered a physiological substrate for myeloperoxidase (MPO) and its effect on myeloperoxidase-dependent processes is widely attributed to scavenger or quencher actions on hypochlorous acid (Myzak and Carr, 2002; Savenkova et al., 1994). Polymorphonuclear cells such as neutrophils present millimolar concentrations of ascorbic acid in their cytosol which can be understood as an important protective role against the action of reactive species produced by neutrophils (Guaiquil et al., 2001; Wang et al., 1997). Deficiency of this vitamin is associated with impaired function of this cell type, including the reduction of its antimicrobial activity (Goldschmidt, 1991) and decreased spontaneous apoptosis (Vissers and Wilkie, 2007). Because both antioxidants are present in specific microenvironment in cells compartments, we believe that a combination of astaxanthin with vitamin C can improve the antioxidant effect of both.

The purpose of the present study was to find out whether co-treatment of human neutrophils with high glucose (20 mM) and MGO can alter the biochemical parameters of these immune cells. High glucose was used as a physiological intracellular source of MGO as previously described (Dhar et al., 2008). We also examined if astaxanthin associated with vitamin C can improve those biochemical parameters. In addition, we evaluated the mechanism underlying this modulation.

2. Materials and methods

2.1. Reagents

Methylglyoxal, D-glucose, astaxanthin, dihydroethidium, vitamin C, propidium iodide and most of the other chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), except RPMI-1640 culture medium, lucigenin and pluronic acid, and acetoxymethylester (Fura-2AM), which came from Invitrogen (CA, USA). Common reagents for buffers (e.g. PBS) and regular laboratory solutions were obtained from Labsynth (Diadema, SP, Brazil).

2.2. Subjects

The Ethical Committee of the Universidade Cruzeiro do Sul approved the experimental procedure of this study. Around 30 healthy adult women and men (mean age 21.0 ± 4.0) were included in the present study. The subjects recruited did not present any systemic or topical therapeutic regimen, a smoking history, alcohol habits, obesity or any other systemic diseases at least for the last 2 months (based on an anamnesis protocol).

2.3. Cell isolation and culture condition

Neutrophils were obtained through the collection of human peripheral blood by venipuncture procedure in vacuum/siliconized tubes containing 0.1 mM EDTA. Peripheral blood neutrophils were isolated under sterile conditions by using a density gradient present in the reagent Histopaque 1077 (Sigma–Aldrich), according to the manufacturer's instruction. After obtained, neutrophils were counted in a Neubauer chamber using Trypan blue (1%). Neutrophils (1×10^6/mL) from each volunteer were cultured in 1 mL of RPMI-1640 medium supplemented with 10% fetal bovine serum, 20 mM Hepes, 2 mM glutamine, and antibiotics (streptomycin 100 units/mL and penicillin 200 units/mL) or ressuspended in Tyrode's solution (137 mM NaCl, 2.68 mM KCl, 0.49 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.6 mM D-glucose, and 5 mM acid HEPES, pH 7.4) for acute assays.

Before starting our experiments we evaluated the toxicity of increasing concentrations of MGO on neutrophils. For this purpose, cells (2.5×10^5) were treated for 18 h with MGO in concentrations ranging from 1 to 500 μM. After this period the cells were collected and analyzed by Trypan blue exclusion. In the literature, the

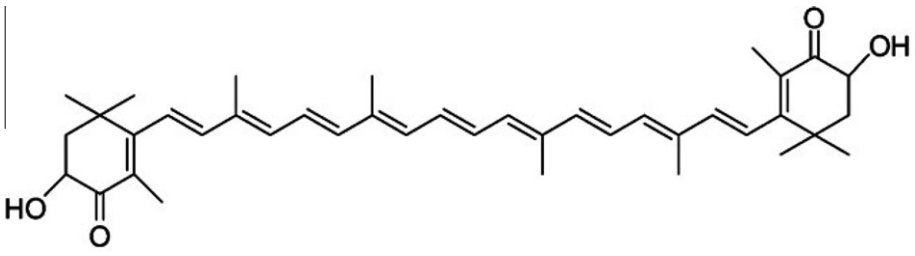

Fig. 1. Chemical structure of astaxanthin.

physiological concentration of MGO in plasma is about 5 μM, but levels can be 5–6 times higher in patients with diabetes types 1 and 2 (Dutra et al., 2005). Based on those data, the concentration of MGO selected to be used in the present study was 30 μM MGO (nontoxic, data not shown) in Tyrode's solution. Glucose concentration was used at 20 mM, also confirmed as a nontoxic concentration (Trypan blue exclusion, data not shown). Astaxanthin at 2 μM was solubilized in DMSO, whereas vitamin C at 100 μM was solubilized in Tyrode's solution. The following experimental groups were created: control (without treatment), AV (astaxanthin + vitamin C), GM (glucose + methylglyoxal) and AVGM (astaxanthin + vitamin C + glucose + methylglyoxal). Cells were cultured at 5% CO_2 for 18 h at 37 °C and then were collected, centrifuged and stored at −80 °C to assay glutathione content and antioxidant enzyme activity. To measure cytokines release, cells were cultured for 18 h and the supernatant was collected and stored under the same condition. ROS production and phagocytic capacity were assayed in neutrophils after acute treatment of cells.

2.4. Cell membrane integrity

To assess whether the concentration of MGO, glucose and both antioxidants astaxanthin and vitamin C selected for the experiments caused toxicity in neutrophils, we assayed cell viability by using flow cytometer analysis. Immediately after being obtained and at the end of the culture period (24 h), cells (5×10^5) were treated as previously described and then used to test the membrane integrity. This assay was carried out in a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA) using propidium iodide (PI) (50 μg/mL) dissolved in phosphate buffered saline (0.137 M NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, pH 7.4). PI is a highly water-soluble fluorescent compound that cannot pass through intact membranes and is generally excluded from viable cells. When cells lose membrane integrity it passes through membrane and binds to DNA. Therefore, an increase in fluorescence to PI indicates a decrease in the proportion of viable cells. Fluorescence of PI was determined in FL2 channel (orange-red fluorescence-585/42 nm). The results were expressed as percentage of the control group.

2.5. Neutrophil functional parameters

2.5.1. Phagocytic capacity

Neutrophils (5×10^5 cell/well) were treated and incubated for 60 min at 37 °C in 1 mL RPMI 1640 medium with opsonised zymosan particles. Zymosan particles (5×10^6/well) were opsonized by incubation in the presence of control serum for 60 min. Afterwards cells were harvested, citocentrifuged, stained and counted in an optical microscope. The score of phagocytosis was expressed by the number of cells that had one, two, three, four or more phagocyted zymosan particles (Sampaio et al., 2001).

2.5.2. Measurement of hypochlorous acid (HOCl) production

Production of HOCl by neutrophils was evaluated according to the method described by Dypbukt et al. (Dypbukt et al., 2005). In short, after treatment neutrophils (6×10^5/well) were stimulated with phorbol myristate acetate (PMA) (60 ng/well) for 60 minutes. The reaction was performed in a modified PBS (NaCl 140 mM, KCl 10 mM, $MgCl_2$ 0.5 mM, $CaCl_2$ 1 mM, glucose 1 mg/mL and taurine 5 mM), pH 7.4. Reactions were stopped by the addition of 26.8 units/mL of catalase. Cells were then centrifuged, the supernatant (200 μL) was collected and added with 50 μL of solution containing 2 mM of 3,30,5,50-tetramethylbenzidine (TMB), 100 μM sodium iodide, and 10% dimethylformamide in 400 mM acetate buffer. After 5 min, absorbance was recorded at 650 nm in a microplate reader and a standard curve (1–40 μM of HOCl) was used to determine the concentration of hypochlorous acid.

2.5.3. Measurement of myeloperoxidase (MPO) release from neutrophils

The measurement of MPO enzyme activity was performed by oxidation of luminol in the presence of H_2O_2 and PMA according to Hatanaka et al. (2006). Neutrophils (2×10^6 cells/well) were exposed for 30 min, at 37 °C, with or without 2 μM of astaxanthin; 100 μM of vitamin C and/or 20 mM of glucose, and 30 μM of MGO in the presence or absence of PMA. After incubation, the medium was immersed into ice and centrifuged at 500*g* for 10 min, at 4 °C, to separate the supernatant from the cells. The supernatant was used to measure MPO activity. The reaction was run in PBS, H_2O_2 (0.1 mM) and luminol (1 mM), at 37 °C, in a final volume of 300 μL. Chemiluminescence was determined in a microplate reader. Results are expressed as relative luminescence unit (RLU) of degranulation.

2.5.4. Glucose-6-phosphate dehydrogenase (G6PDH) activity

Glucose-6-phosphate dehydrogenase (G6PDH), EC 1.1.1.49, is a key regulatory enzyme of the oxidative segment of the pentose-phosphate pathway. It produces equivalent reducing agents in the form of NADPH to meet some cellular needs for reductive biosynthesis and as a contribution to the maintenance of the cellular redox state (Costa Rosa et al., 1995). The maximum activity of this enzyme was previously described (Guerra and Otton, 2011). The extraction buffer consisted of Tris-HCl (50 mM), EDTA (1 mM) at pH 8.0. The reaction buffer used contained Tris-HCl (86 mM), $MgCl_2$ (6.9 mM), NADP+(0.4 mM), glucose-6-phosphate (1.2 mM) and Triton X-100 0.05% (v/v) at pH 7.6. The total volume of the sample was 374 μL. The reaction was started by adding glucose-6-phosphate to the medium. The absorbance at 340 nm was analyzed in a microplate reader (Tecan, Salzburg, Austria), and the results are expressed as nmol/min/mg of protein.

2.5.5. Release of pro-inflammatory cytokines

Cytokines IL-6, IL-1β and TNF-α were assayed in cell culture supernatant with ELISA kits according to the manufacturer's instructions (Quantikine, R&D System, Minneapolis, MN, USA). Neutrophils (1×10^6/mL) were cultured for 18 h in the presence or absence of LPS as a stimulus (10 μg/mL). Afterwards, cells were centrifuged (1000*g*, 4 °C, 10 min) and the supernatant was collected and stored at −80 °C until they are used for cytokines determination. The lower limits of detection for the ELISA analyses were as follows: 1.17 pg/mL for IL-6 and 1.95 pg/mL for IL1-β and TNF-α.

2.6. Oxidative parameters

2.6.1. Dihydroethidium assay

Dihydroethidium (DHE) is a fluorescent probe used to measure intracellular superoxide anion production. Once inside the cell, DHE is rapidly oxidized to ethidium (a red fluorescent compound) by superoxide and/or H_2O_2 (in the presence of peroxidase). Neutrophils (5×10^5/well) were incubated with 5 μM DHE for 15 min at room temperature in the dark. Afterwards, the cells were treated and stimulated with PMA (20 ng/well). As a internal control, cells were treated with either 10 μM DPI or 5 μM rotenone (a complex 1 – electron transport chain inhibitor), and 0.4 mM sodium azide (SA), a complex III – electron transport chain inhibitor for 30 min prior to treatment. Also, to ensure the specificity of DHE to superoxide anion, hydrogen peroxide (50 μM) was added to control-PMA stimulated cells. The fluorescence was analyzed in a microplate reader (Tecan, Salzburg, Austria) (396 nm wavelength excitation and 590 nm wavelength emission). The results were expressed as percentage of the control group.

2.6.2. Lucigenin

The lucigenin chemiluminescent probe was utilized to measure the extracellular superoxide anion content mainly produced

through NADPH-oxidase activation. Lucigenin releases energy in the form of light after excitation by superoxide anion. The chemiluminescence produced was monitored by a luminometer for 60 min (Tecan, Salzburg, Austria). Lucigenin (5 μM) was added to cells (5×10^5/well) treated with or without 20 mM of glucose and 30 μM of MGO, in the presence or absence of 2 μM of astaxanthin, 100 μM of vitamin C in Tyrode's buffer supplemented with fetal bovine serum 1%. The experiments were carried out in triplicate in the presence and absence of opsonized zymosan particles (1×10^6/well) used as a ROS-inducer. As internal control, 10 μM diphenyleneiodonium (DPI), a NADPH-oxidase inhibitor, or 0.4 mM sodium azide (SA), a complex III – electron transport chain inhibitor, were added to control cells 30 min prior to the lucigenin evaluation. Results are expressed as chemiluminescence relative units. The statistical analysis was performed by AUC calculation (area under the curve) of at least three different experiments performed in triplicate.

2.6.3. Hydrogen peroxide production by phenol red assay

Hydrogen peroxide (H_2O_2) production was measured according to Pick and Mizel (1981), based on horseradish peroxidase, which catalyzes the phenol red oxidation by H_2O_2. Neutrophils (5×10^5/well) were incubated with or without 2 μM of astaxanthin, 100 μM of vitamin C and 20 mM of glucose, and 30 μM of MGO in Tyrode's buffer, mixed with 0.28 mM phenol red and horseradish peroxidase (1,000 units/mg) at 37 °C for 1 h. The production of H_2O_2 was measured in the absence and presence of PMA (20 ng/well). The reaction was terminated by alkalinization (addition of 10 μL of NaOH 1 M solution) and absorbance at 620 nm was measured to evaluate H_2O_2 concentration (compared to a standard curve). The results were expressed as percentage of the control group.

2.6.4. General ROS production assayed by DCFH-DA

The probe DCFH-DA is primarily used as an indicator of the production of H_2O_2 (Brandt and Keston, 1965) but it is also described as being oxidized by other ROS such as $HO^{\cdot}$, $ROO^{\cdot}$, NO and peroxynitrite (Crow, 1997). The cells (5×10^5/well) were preloaded with DCFH-DA (5 μM) by incubation in culture medium for 30 minutes. DCFH-DA is cleaved inside the cells by non specific esterase and turns to high fluorescent 2,7-dichlorofluoroscein (DCF) upon oxidation by ROS. After the loading period, cells were treated with or without 2 μM of astaxanthin, 100 μM of vitamin C and 20 mM of glucose, and 30 μM of MGO in Tyrode's buffer for 60 minutes. The experiments were conducted in the presence or absence of PMA (20 ng/well). Afterwards, cells were centrifuged and resuspended in 300 μL of Tyrodés buffer, and the fluorescence was monitored in spectrofluorimeter Tecan (Salzburg, Austria) with excitation at 485 nm and emission at 530 nm. As an internal control 50 μM of H_2O_2 was added to control cells under PMA-stimulation to ensure the specificity of DCFH-DA. The results were expressed as percentage of the control group.

2.6.5. Nitric oxide production ($NO^{\cdot}$)

$NO^{\cdot}$ production was performed according to Ding et al. (1988) through nitrite determination. Nitric oxide is rapidly converted into nitrite in aqueous solutions and, therefore, the total nitrite can be used as an indicator of nitric oxide concentration. The spectrophotometric analysis of the total nitrite content was performed by using the Griess reagent (1% sulfanilic acid, 0.1% *N*-1-naphthylethylenediamine dihydrochloride) in supernatants. Neutrophils (5×10^5/100 μL) in RPMI 1640 medium were treated with or without 2 μM of astaxanthin, 100 μM of vitamin C and 20 mM of glucose and 30 μM of MGO and stimulated with lipopolysaccharide (LPS) at 10 μg/well for 4 h. Then, the same volume of Griess (187 μL) was added to cells and the absorbance was measured in 550 nm. The nitrite concentration was determined using sodium nitrite as a standard (0–60 μM). The results were expressed as percentage of the control group.

2.6.6. Intracellular Ca^{2+} concentration

Changes in cytosolic Ca^{2+} levels were monitored by fluorescence using the calcium-sensitive probe Fura 2-AM (Otton et al., 2007). Neutrophils (1×10^6/well) were treated with or without 2 μM of astaxanthin, 100 μM of vitamin C and 30 μM of MGO in the presence of opsonized zymosan particles (1×10^6/well). Total intracellular release of Ca^{2+} was monitored for 60 min in a microplate reader (Tecan, Salzburg, Austria). Transformation of the fluorescent signal to Ca^{2+} (in nmol Ca^{2+} per minute) was performed by calibration with ionomycin (100 μM, maximum concentration) followed by EGTA addition (60 μM, minimum concentration) according to the Grynkiewicz equation (Grynkiewicz et al., 1985).

2.7. Antioxidant profile of neutrophils

2.7.1. Antioxidant enzyme activity

To evaluate antioxidant enzyme activities as well as GSH and GSSG content, we performed these specific assays after 24 h of culture as previously described. After this period, cells (5×10^6) were harvested, centrifuged and the pellet was added with a specific extraction buffer. Cells were then ruptured by ultrasonication in a Vibra Cell apparatus (Connecticut, USA), centrifuged for 10 min, 10,000*g* at 4 °C and the supernatant was used for analysis.

Superoxide dismutase (SOD), Catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities were determined in neutrophils using a microplate reader (Tecan, Salzburg, Austria). CAT activity was measured as described by Aebi (1984) based on the direct decomposition of hydrogen peroxide (H_2O_2). SOD activity was measured using the method described by Ewing and Janero (1995) which involves the reduction of $O_2^{\cdot-}$ radicals by nitroblue tetrazolium (NBT) for 3 min. Glutathione peroxidase (Mannervik, 1985) and glutathione reductase (Carlberg and Mannervik, 1985) activities were measured based on the oxidation of β-NADPH in the presence of tert-butyl hydroperoxide, used as substrate.

2.7.2. GSH and GSSG content

Reduced (GSH) and oxidized (GSSG) glutathione content in neutrophils were measured as described by Rahman et al. (2006). The method is based on the reaction between reduced thiol groups (such as in GSH) with 5,5́-dithiobis-2-nitrobenzoic acid (DTNB) to form 5-thio-2-nitrobenzoic acid (TNB), which is stoichiometrically detected by absorbance at 412 nm. Purified GSH and GSSG (Sigma-Aldrich) were used as standards.

2.7.3. Protein measurement

The total protein content of cells was measured by the method of Bradford, using BSA as standard (Bradford, 1976).

2.8. Statistical analyses

All data points are presented as the mean values with standard errors of at least three independent experiments, each one performed in triplicate. The data were analyzed by one-way ANOVA followed by the Tukey's post-test. The software employed for statistical analysis was GraphPad Prism (version4; GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Cell membrane integrity

Cell membrane integrity was tested by using flow cytometer and propidium iodide as a probe. After 24 h of culture, none of

the groups showed any significant loss of cell membrane integrity. These results indicate that the concentrations of MGO, glucose, astaxanthin and vitamin C selected to evaluate the functional parameters of neutrophils did not cause cell death (Fig. 2). Additionally, MGO, high glucose, astaxanthin and vitamin C alone did not promote changes in cell viability (data not shown).

3.2. Phagocytic capacity

In order to determine the potential of MGO and glucose to modulate the phagocytic capacity of human neutrophils, we measured the incorporation of opsonized zymosan particles in the cells (Table 1). There was a significant decrease of 30% in the phagocytic capacity of neutrophils after treatment with glucose + methylglyoxal (GM group), whereas there was an increase of 22% in the phagocytic capacity after AV-treatment as compared to the control group. When GM-treated cells were added with antioxidants (AVGM group) we observed a complete restoration in the phagocytic capacity. Neither glucose nor MGO alone promoted the same effect observed when those compounds were combined (data not shown). Vitamin C alone promoted improvement in the phagocytic capacity (data not shown).

3.3. Myeloperoxidase (MPO) activity

MPO activity was evaluated in neutrophils after induction of neutrophil-degranulation by addition of PMA for 30 min (Table 1). As compared with the control group, MPO activity was increased by 40% in the GM-group and reduced 86% and 94% in the AV and AVGM groups, respectively.

3.4. Hypochlorous acid production

Neutrophils were stimulated to produce hypochlorous acid by the addition of PMA (60 ng/well). Hypochlorous acid concentration was significantly reduced by 25% in the AV group and increased by 135% and 99% in the GM and AVGM groups, respectively, when compared with the control group (Table 1).

3.5. Glucose-6-phosphate dehydrogenase activity

The maximum G6PDH activity was assessed by the reduction of the co-factor $NADP^+$ into NADPH in human neutrophils (Table 1). GM promoted a significant reduction of 37% in G6PDH activity and astaxanthin + vitamin C addition (AVGM group) increased the G6PDH activity by 52% when compared to the GM group.

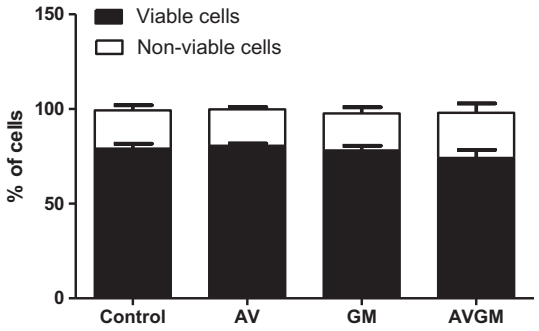

Fig. 2. Viability of human neutrophils (5×10^5/well) after 24 h of culture. Cells were treated with glucose (20 mM) and MGO (30 μM) with or without the antioxidants astaxanthin (2 μM) and vitamin C (100 μM), and cultured as previously described. Afterwards, cells were harvested and then analyzed by flow cytometry using propidium iodide as a probe. Results are presented as mean ± SEM of at least three independent experiments, each one performed in triplicate.

3.6. Cytokines Release

TNF-α, IL-1β, and IL-6 are inflammatory cytokines which play important roles in immune responses to a variety of inflammatory stimuli. Therefore, we evaluated the effects of GM on TNF-α, IL-1β, and IL-6 after 18 h of LPS-stimulation. The levels of these cytokines in the culture supernatants were measured using ELISA kits. Control neutrophils treated with LPS showed a significant increase in cytokine production when compared with the basal condition (100 ± 10 pg/ml, data not shown). The production of pro-inflammatory cytokines IL-6, IL-1β and TNF-α by human neutrophils in the AVGM group was significantly decreased by 46%, 36% and 77%, respectively, when compared with the GM-group. IL-1β and TNF-α were also reduced in the AV-group by 42% and 89%, respectively, when compared with the control group.

3.7. Measurement of superoxide anion production

The production of reactive oxygen species is among the key weapons used by neutrophils to exterminate pathogens. In order to evaluate some possible modulation of MGO + glucose and astaxanthin and vitamin C in a few of these species we used different probes. Superoxide anion production was measured by using two different probes, DHE and lucigenin. As assayed by the DHE probe, when GM-treated cells were stimulated with PMA there was an increase of 41% in the superoxide anion production compared with the PMA-control cells. Cells treated with astaxanthin plus vitamin C decreased production of superoxide anion by 54% as compared with the control-stimulated group. Addition of antioxidants to cells treated with GM (AVGM group) promoted a reduction of 66% in superoxide as compared with the GM group in stimulated conditions. Rotenone + Sodium Azide and DPI were added to neutrophils under PMA-stimulation. Both inhibitors significantly reduced superoxide anion production to basal levels. SOD enzyme addition was used to evaluate the specificity of DHE probe to superoxide anion (Fig. 3A), and as expected there was no significant fluorescence in this group. As an internal control, we also carried out the addition of 50 μM of H_2O_2 to PMA-treated cells. As expected, there was no increase in the fluorescence produced, thus ensuring the specificity of DHE for superoxide anion (data not shown).

The lucigenin probe (Fig. 3B) was used to measure the activation of NADPH-oxidase and then extracellular superoxide anion production. For this purpose neutrophils were challenged with opsonized zymosan particles. All treatments promoted a reduction in the superoxide anion production as compared with control-zymosan group. DPI (10 μM) addition 30 min before the treatment with zymosan particles promoted a total inhibition in the lucigenin signal, indicating that, indeed, superoxide anion production occurred via NADPH-oxidase activation. Sodium azide (SA) did not promote a significant reduction in the lucigenin light emission, indicating the specificity of lucigenin probe to superoxide anion present in the extracellular compartment.

3.8. Hydrogen peroxide production

Hydrogen peroxide production was evaluated by the method of phenol red oxidation (Fig. 3C) and DCFH-DA probe (Fig. 3D). MGO + glucose did not promote any modification in the H_2O_2 production. However, in both assays when the neutrophils were treated with the antioxidants in the AV and AVGM groups, there was a

Table 1
Functional parameters of human neutrophils.

	Control	AV	GM	AVGM
Phagocytosis (Score of phagocitic capacity)	263 ± 11	319 ± 11[a]	174 ± 11[a]	315 ± 9[b]
MPO activity (relative luminescence unit)	10516 ± 688	670 ± 61[a]	14730 ± 588[a]	1456 ± 106[a,b]
HOCl production (μM of HOCl/6 × 10^5 cells)	18.8 ± 1.22	14.1 ± 0.66[a]	44.2 ± 0.53[a]	37.5 ± 1.52[a,b]
G6PDH activity (nmol/min/mg/protein)	48.6 ± 5.52	42.5 ± 5.33	35.1 ± 4.05[a]	53.3 ± 3.34[b]
IL-6 (pg·ml^{-1})	428 ± 71	420 ± 43	669 ± 59[a]	360 ± 56[b]
IL-1β (pg·ml^{-1})	121 ± 12	71 ± 5[a]	103 ± 8	66 ± 5[a,b]
TNF-α (pg·ml^{-1})	69 ± 6	8 ± 1[a]	53 ± 4	11 ± 1[a,b]

The results are presented as mean ± SEM of at least three experiments performed in triplicate. In the assay of cytokines production, all groups were LPS-stimulated and in the myeloperoxidase assay and hypochlorous acid production, both experiments were stimulated with PMA.
[a] Compared to control group ($p < 0.001$).
[b] Compared to GM group ($p < 0.0001$).

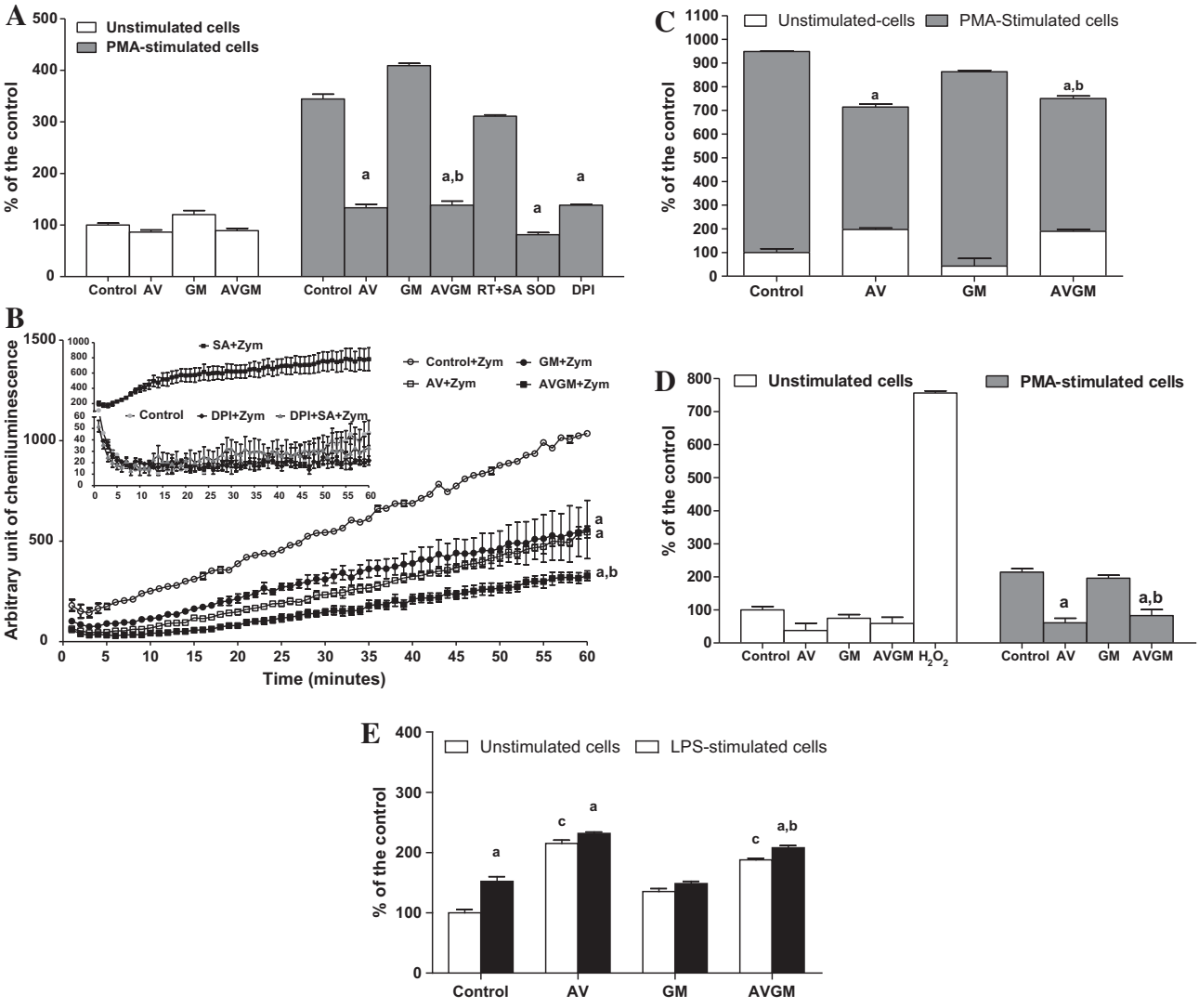

Fig. 3. (A) Intracellular superoxide anion production assayed by DHE probe, (B) extracellular superoxide anion production assayed by lucigenin probe, (C) hydrogen peroxide production assayed by phenol red oxidation, and by using DCFH-DA probe (D), NO production assayed by Griess reagent (E). Neutrophils were stimulated as described in the Material and Methods section. The results are presented as mean ± SEM of at least three independent experiments, each one performed in triplicate. (a) compared to control-stimulated group ($p < 0.0001$); (b) compared to GM-stimulated group ($p < 0.001$); (c) compared to control non-stimulated group ($p < 0.001$).

significant reduction in the production of hydrogen peroxide after PMA-stimulation. As a positive control for the DCFH-DA probe we added 50 μM of H_2O_2. Our data show that the DCFH-DA probe has a high specificity to hydrogen peroxide.

3.9. Nitric oxide production

The $NO^{\bullet}$ production was evaluated in cells at basal and LPS-stimulated conditions (Fig. 3E). In basal conditions there was

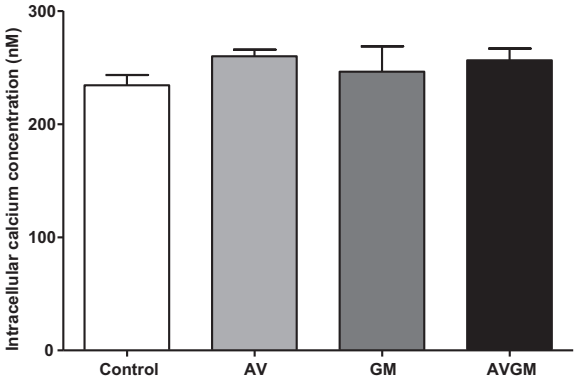

Fig. 4. Total intracellular calcium [Ca^{2+}]i (nM) mobilization monitored during 20 min. Cells (1×10^6/well) were previously loaded with 5 μM Fura 2-AM for 1 h and then incubated with glucose + MGO and/or astaxanthin + vitamin C and stimulated with opsonized zymosan particles (5×10^6/well). The results are presented as mean ± SEM of at least three independent experiments, each one performed in triplicate.

an increase of 115% and 88% in the AV and AVGM groups when compared with the control group. After LPS-stimulation there was an increase in $NO^{\cdot}$ production of 52% and 37% in the AV and AVGM groups respectively, as compared with the control group.

3.10. Intracellular calcium concentration

Intracellular calcium mobilization was monitored for 20 minutes by using Fura 2-AM probe in neutrophils challenged with opsonized zymosan particles (Fig. 4). There was no significant difference in calcium release among all groups.

3.11. Activity of antioxidant enzymes

Total SOD activity was decreased in the GM and AV groups by 28% and 23%, respectively, as compared to the control group (Table 2). In the AVGM group there was an increase of 35% in the total SOD activity in comparison with the GM group. Maximum activity of catalase (CAT) increased in 43% in the GM group, whereas there was a reduction of 32% and 17% in the AV and AVGM groups, respectively, both compared with the control group. In the AVGM treated cells we observed a reduction of 42% when compared with the GM group. However, there was a 3-fold increase in the GPx activity in the AVGM group compared with the control. GM and AV reduced the GR activity in 82% and 25%, respectively, compared to the control group, whereas in the AVGM group there was an 11-fold increase in GR activity compared to the GM group (Table 2).

3.12. GSH and GSSG content and GSH/GSSG ratio

The content of GSH increased 93% after addition of antioxidants in the AV group when compared with the control group. As a consequence, GSH/GSSG ratio was increased in the AV group when compared with the control group (Table 2).

4. Discussion

Diabetic patients suffer from many common infections whose causes remain unknown. One hypothesis suggests that the damage would be caused by immune glycation which can be responsible for increased susceptibility to infections (Price et al., 2010). Among the glycation agents we call attention to methylglyoxal, which is a dicarbonyl reactive that originates from the breakdown of glucose (Desai and Wu, 2007). The results of this study showed that co-treatment of human neutrophils with MGO/high glucose promoted important modifications in the neutrophil function *in vitro*. Treatment of neutrophils with MGO/high glucose did not promote citotoxicity; however, it reduced the phagocytic capacity and the G6PDH, total/SOD and GR activities. Additionally, there was an increase in the activity of myeloperoxidase (MPO) with consequent increase in the hypochlorous acid production, CAT activity and in the release of IL-6 cytokine without changes in intracellular calcium mobilization. Contrasting with other studies (Dhar et al., 2008), MGO/high glucose did not show a strong pro-oxidant effect, as demonstrated by the ratings in the production of superoxide anion, hydrogen peroxide and nitric oxide. These results indicate which MGO/high glucose effects did not involve oxidative stress or calcium release. In addition, our study shows that the association of astaxanthin with vitamin C greatly improved neutrophil phagocytic capacity, decreasing all reactive oxygen species measured, pro-inflammatory IL-1β and TNF-α release, MPO activity and HClO production. The combination of astaxanthin with vitamin C alone has more antioxidant and anti-inflammatory than when they were in the presence of MGO/high glucose.

The abnormal glucose homeostasis in diabetes due to the formation of the highly reactive metabolite MGO (Fleming et al., 2011; Tajima et al., 2002; Thornalley, 2005) may be the key step in triggering the neutrophil dysfunction. Neutrophils are the first immune cells to enter the site of infection or injury and there neutrophils kill microorganisms by ingesting them into phagocytic vacuoles (phagosomes). Therefore, phagocytosis is undoubtedly one of the most important roles of neutrophils. During phagocytosis, granules in the cytoplasm of neutrophils merge with the newly formed phagosome, forming the phagolysosome (Kuijpers et al., 2001). The cytoplasmic granules of neutrophils have as one of their main constituent myeloperoxidase, the enzyme that catalyzes the reaction of hydrogen peroxide in the presence of halide ions such as chloride, bromide and iodide hipohalosos acids, in particular hypochlorous acid (Hampton et al., 1998; Kettle et al., 1997). Hypochlorous acid is considered one of the most important antimicrobial agents produced by neutrophils. During phagocytosis there is activation of the NADPH oxidase, an enzyme complex that assembles in the phagosomal membrane and converts oxygen into the superoxide radical anion ($O^{2\cdot-}$). Superoxide anion is generated in the external surface (*i.e.* inside the phagosome) with reducing equivalents supplied by intracellular NADPH. During granules secretion, phagocytosis and killing of pathogens, levels of calcium in the cytosol are usually increased (Lee et al., 2003).

In cells co-treated with MGO/high glucose (GM group) there was an increase in MPO enzyme activity and consequently in the production of hypochlorous acid (Table 1), whereas neither high glucose nor MGO alone yielded the same effect (data not shown). Myeloperoxidase is an enzyme stored in azurophilic granules of polymorphonuclear neutrophils and released into extracellular fluid during inflammatory processes. Several studies have shown its involvement in oxidative stress and inflammation. Recently, MPO has been considered as a possible marker of plaque instability and a useful tool for the prognostic evaluation of patients with coronary artery disease (Gustapane et al., 2011). Possibly, increased release and activity of MPO in neutrophils promoted by co-treatment of neutrophils with MGO/high glucose could contribute to the development of the micro- and macro-vascular complications observed in diabetic condition.

In contrast, cells treated with antioxidants promoted a marked reduction in the MPO and HClO production along with a drastic reduction in all reactive oxygen species. This effect was not observed when cells were treated with either astaxanthin or vitamin C alone. Superoxide is a physiological substrate for MPO and their interactions are central to an important host defense mechanism. When released by neutrophils, MPO enzyme operates in the

Table 2
Antioxidant enzyme activities and glutathione content.

	Control	AV	GM	AVGM
Total/SOD (U/mg protein)	18.48 ± 1.07	14.31 ± 0.91[a]	13.36 ± 0.37[a]	18.09 ± 0.33[b,c]
CAT (μmol/min/mg protein)	40.51 ± 1.18	27.47 ± 1.04[a]	57.95 ± 1.40[a,b]	33.76 ± 0.98[a,b]
GPx (mU/mg protein)	4.54 ± 0.79	3.30 ± 0.58	6.19 ± 0.61	13.99 ± 1.09[a]
GR (mU/mg protein)	31.03 ± 2.06	23.35 ± 3.09[a]	5.55 ± 1.23[a]	64.49 ± 6.17[a,b]
GSH (μM of GSH/mg of protein)	2.78 ± 0.37	5.66 ± 0.69[a]	1.13 ± 0.08	1.88 ± 0.34[c]
GSSG (μM of GSH/mg of protein)	0.37 ± 0.04	0.43 ± 0.03	0.24 ± 0.05	0.32 ± 0.07
GSH/GSSG (μM of GSH/mg of protein)	6.37 ± 0.81	13.11 ± 1.38[a]	5.25 ± 1.01	6.76 ± 1.34[c]

The results are presented as mean ± SEM of at least three experiments performed in triplicate.
[a] Compared to the control group ($p < 0.001$).
[b] Compared to the MG group ($p < 0.0001$).
[c] Compared to the AV group ($p < 0.001$).

presence of a flux of superoxide. Winterbourn and Kettle (2004) showed that superoxide has a profound influence on oxidative reactions catalysed by MPO. It reacts directly with the enzyme to modulate production of hypochlorous acid. Within neutrophil phagosomes, where MPO acts by killing micro-organisms, it may be the preferred substrate for the enzyme. Superoxide also reacts rapidly with radicals generated by MPO forming different species. These species are likely to be toxic and contribute to the pathophysiological actions of MPO (Winterbourn and Kettle, 2004). Therefore, reduced superoxide anion and hydrogen peroxide production promoted by astaxanthin and vitamin C can be involved in the reduced MPO and HClO production as well as a direct scavenger effect promoted by antioxidants.

In addition, the phagocytic capacity of neutrophils and G6PDH activity, key enzyme of pentose pathway involved in NADPH formation, were decreased in cells after treatment with MGO/high glucose. A decrease in the phagocytic capacity accompanied by a decrease in NADPH availability could mean minor neutrophil effectiveness to destroy pathogens. This fact has been associated with the impairment in neutrophil function observed in diabetes (Lecube et al., 2011). Decreased phagocytic capacity by induced by MGO/high glucose was prevented by treatment of cells with the combination of antioxidants astaxanthin and vitamin C. Previous studies from our group also showed that addition of astaxanthin alter the capacity of neutrophils to phagocytose opsonized zymosan particles under different conditions (Guerra and Otton, 2011; Macedo et al., 2010; Marin et al., 2011). The mechanism by which the antioxidant astaxanthin improves phagocytic capacity of neutrophils remains to be elucidated in future studies. Although it is well known that phagocytosis in neutrophil cells is a process which involves intracellular calcium mobilization, in the present study we did not observe any changes in intracellular calcium concentration among all groups.

By means of Maillard reaction, MGO is able to cross-link with cellular proteins on targeted amino acids (arginine, lysine), leading to the formation of advanced glycation end-products (AGEs), and thus contributing to aging and complications in chronic diseases (Fleming et al., 2011; Thornalley, 2005). Similarly to our results, some authors showed which MGO inactivate the enzyme glutathione reductase (Paget et al., 1998; Park et al., 2003; Wu and Juurlink, 2002). Glutathione reductase recycles GSSG using NADPH as a cofactor, reestablishing the intracellular content of reduced glutathione (GSH) (Juurlink, 1999; Wu and Juurlink, 2002). Other studies have shown that MGO reduced GSH content making cells more sensitive to oxidative stress (Kikuchi et al., 1999; Meister, 1988; Shinpo et al., 2000). The inactivation of MGO is a process catalyzed by the glyoxalase system that uses glutathione (GSH) as a cofactor. MGO inactivated bovine glutathione peroxidase in a time and dose-dependent manner, forming a connection with glutathione to sites of arginine 184 and 185 (Park et al., 2003). High concentration of MGO in plasma and aorta are associated with increased levels of superoxide, significantly reduced levels of GSH, decreased activity of glutathione peroxidase and glutathione reductase in SHR rats with high blood pressure (Wang et al., 2005). Contrasting with these studies, we did not observe any change in the content of GSH, GSSG and in the rate GSH/GSSG (Table 2).

Studies by Chang and colleagues (Chang et al., 2005) demonstrated that MGO caused mitochondrial oxidative stress by increasing the mitochondrial production of superoxide, nitric oxide and peroxynitrite. MGO can inhibit complex III and thereby disrupt the electron transport chain, leading to leakage of electrons to form superoxide anion (Wang et al., 2009). The direct effect of MGO on mitochondria was investigated by Desai and colleagues (Desai and Wu, 2007) using MitoSOX, a mitochondrial specific probe used to detect mitochondrial superoxide production. Incubation of vessel smooth muscle cells with MGO 30 μmol/L significantly induced mitochondrial superoxide production as compared with the group of untreated cells. Another study showed that incubation of vascular smooth muscle cells (VSMCs) of rat aorta with MGO significantly increased production of superoxide in a dose-dependent manner, which was prevented by the addition of enzyme superoxide dismutase (SOD) or inhibition of NADPH oxidase with DPI (Chang et al., 2005). MGO also increased the generation of hydrogen peroxide in VSMCs and increased formation of peroxynitrite (ONOO–) through the induction of inducible NOS (iNOS) (Chang et al., 2005). Similar results were found by Ward and McLeish, who added MGO in neutrophils and found that there was a significant increase in basal production of hydrogen peroxide and superoxide anion in a dose-dependent manner of the MGO concentration, indicating increased respiratory burst activity (Ward and McLeish, 2004). The effect of MGO was significantly higher in platelets pretreated with an agent that depletes GSH and glutathione peroxidase (Leoncini and Poggi, 1996). Contrasting with these results, our data show that MGO/high glucose did not cause any major change in the production of reactive oxygen/nitrogen species in neutrophils (Fig. 3).

One acceptable reason for the weak pro-oxidant effect of MGO/high glucose could be the MGO concentration used in the present study. Many authors demonstrate a modulation of MGO on different cell types using high MGO concentrations ranging from 100 μM to 1 mM (Chang et al., 2005; Desai et al., 2010; Wang et al., 2009). We used MGO at 30 μM, which is considered by some authors a high concentration usually found in the diabetic plasma (Dutra et al., 2005). In addition, the incubation time of neutrophils which MGO/high glucose could be short to promote any permanent modification in the neutrophil function. Several authors have shown that, to be effective as a glycation agent, MGO needs to be incubated for long periods, which was not observed in this work, due to the short half-life of neutrophils in culture.

On the other hand, association of astaxanthin with vitamin C promoted a clear antioxidant effect (Fig. 3) as observed by the marked reduction in the production of superoxide anion and hydrogen peroxide production. Compared with a previous study

from our group that showed a weak astaxanthin antioxidant-effect (Bolin et al., 2010; Campoio et al., 2011; Guerra and Otton, 2011; Macedo et al., 2010), the association of both antioxidants allowed a great antioxidant action. Many authors have reported the effective antioxidant action of either astaxanthin or vitamin C alone, but not in combination. In our model, the astaxanthin/vitamin C system mimics the recycling system of vitamin C/vitamin E. Astaxanthin provides cell membranes with potent protection against free radicals or other oxidative attack. Experimental studies confirm that this nutrient has a large capacity to neutralize free radicals or other oxidant activity in the nonpolar (''hydrophobic'') zones of phospholipid aggregates, as well as along their polar (hydrophilic) boundary zones (Fassett and Coombes, 2011). Vitamin C, in turn, promotes antioxidant effects mainly in water-phase microenvironment.

Neutrophils are capable of expressing a variety of proteins involved in inflammation and immune responses, including cytokines such as IL-1β, IL-1ra (interleukin receptor antagonist), IL-8, IL-12, TNF-α, TGF -β, MIP-1α, MIP-1β, GM-CSF and IFN-α (Scapini et al., 2000). Although cytokines induce pathology when expressed inappropriately, they play important roles in a variety of physiological processes. Wang and colleagues demonstrated that 30 μmol/L MGO for 12 h significantly increased the secretion of pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-8 and tumor necrosis factor (TNF-α), and induced apoptosis in neutrophils (Wang et al., 2007). In this study, MGO/high glucose increased IL-6 production in cells stimulated with LPS. When antioxidants were added to MGO/high glucose treated cells (AVGM group), there was an important reduction in all pro-inflammatory cytokines when compared to the GM group.

In summary, our results show that treatment of neutrophils with high glucose and MGO promotes an injury to the function of neutrophils, and this process appears not to involve oxidative stress or calcium release. In addition, when cells were treated with the association of antioxidants astaxanthin and vitamin C, we observed a significant improvement in the function of neutrophils and in the redox status. The use of antioxidants to prevent or reverse diabetic complications seems to be necessary; however, a single substance cannot achieve this effect. Therefore, we are proposing a combination of two substances that act differently in cell microenvironment, working in a collaborative way. The collaborative way in which the antioxidants work was evidenced in almost all experiments performed as compared with cells treated with antioxidants alone. In the near future, the combination of antioxidants astaxanthin and vitamin C might act as an adjuvant therapy for the treatment of a variety of diseases, including diabetes mellitus. The answer to the question of whether the *in vitro* neutrophils protection achieved by this combination of therapy can be translated to subjects with diabetes will have to wait until completion of the ongoing clinical trial.

Conflict of interest statement

All the authors of the present manuscript declare that there is no any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence our work.

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